Identification of a Synthetic Peptide as Part of a Major Neutralization Epitope of Respiratory Syncytial Virus

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SUMMARY

A 7000 M subunit that carries the major neutralization epitope has been identified by chemical and enzymatic cleavage of the fusion protein of respiratory syncytial (RS) virus (Long strain) with an efficient RS virus-neutralizing monoclonal antibody. Based on the published mRNA-deduced sequence of the A2 strain, coupled to the hydropathicity profile and prediction of protein conformation, the neutralization epitope has tentatively been localized on the first third of the F1 protein N-terminal, probably in the region of amino acids 215Ser to 236Glu. Analysis of three peptides covering different portions of the 212Cys to 236Glu region of the F1 fusion protein identified a peptide (Cys•216Ash to 236Glu) that reacted strongly with the neutralizing monoclonal antibody and that was efficient in blocking neutralization and in plaque-reducing assays, confirming that the neutralization epitope was localized in that region. Further analysis with two other synthetic peptides (212Cys to 222Glu and Cys•221Ile to 236Glu) indicated that the dodecapeptide Ile-Glu-Phe-Gln-Lys-Asn-Asn-Arg-Leu-Leu-Glu mimicked either the whole or a major part of the neutralization epitope. This opens a promising avenue for the simple design of a synthetic peptide vaccine to control RS virus infection.

INTRODUCTION

The genus Pneumovirus of the Paramyxoviridae family consists of human and bovine strains of respiratory syncytial (RS) virus and pneumonia virus of mice. Human RS virus, an important pathogen in young children (Belshe et al., 1984), is thought to be a monotypic strain though minor differences in cross-neutralization assays have been noted. Using monoclonal antibodies (MAbs), Mufson et al. (1985) have been able to separate different isolates into subgroups A and B; however, studies by Prince et al. (1985) suggest that antigenic differences detected in vitro disappear when virus strains are compared in vivo. Recently, using a neutralizing MAb produced against the Long strain of RS virus, a major and highly conserved neutralization epitope was identified which was present on human, bovine and caprine strains of RS virus but not on pneumonia virus of mice. This epitope is localized on the F1 fragment of the fusion glycoprotein (Trudel et al., 1986, 1987).

The fusion glycoprotein is found as a 125K M dimer on the viral envelope and is involved in cell fusion leading to the formation of syncytia and cell penetration. It is composed of two disulphide-linked subunits: F1 with a reported M, varying from 43K to 50K and F2 from 19K to 25K (Walsh et al., 1985, 1986; Trudel et al., 1986).

Expression by recombinant vaccinia viruses of the major and fusion glycoproteins of RS virus (Olmsted et al., 1986) confirmed previous work carried out with MAbs and purified solubilized proteins showing the predominant role of the fusion glycoprotein in inducing immunity.
This paper presents data derived from peptide mapping and amino acid analysis indicating that the major neutralization epitope is located between amino acids 164Glu to 236Glu of the mRNA-deduced sequence of the A2 strain of human RS virus. Synthetic peptides prepared to this region identify the sequence 221Ile to 232Glu as the probable and most likely epitope reacting with our highly specific and RS virus-neutralizing MAb.

**METHODS**

**Cells and virus.** The Long strain of human RS virus (ATCC VR-26; American Type Culture Collection) was propagated on HEp-2 cells (ATCC CCL-23), which were maintained in equal parts of Eagle’s MEM and medium 199 supplemented with 50 μg/ml of gentamicin and 5% foetal calf serum. Infected cells were inoculated at an m.o.i. of 3 and the virus was harvested in the supernatant 27 h post-infection, when the cell monolayer was composed of cells which were completely fused in a giant syncytium. Supernatant was concentrated by precipitation with 6~ polyethylene glycol 6000 Mr and the concentrated virus was purified on a discontinuous 30 to 50 ~ sucrose gradient (Beckman SW28 rotor, 25000 r.p.m., 3 h at 4 °C). Virus fractions were identified by electron microscopy and samples were snap-frozen in liquid nitrogen and stored at -70 °C for further studies.

**Neutralization assays.** Two different microneutralization assays without complement were used. In the first series of assays, 25 μl of twofold dilutions of antisera were mixed with 25 μl of viral suspension containing 100 p.f.u. [plaque- (or syncytia)-forming units] before inoculation of HEp-2 cells and incubation for 5 days in a CO2 incubator: the titre of the antiserum was read as the last dilution which allowed 50% residual infectivity.

To assay for the blocking activity of synthetic peptides, twofold serial dilutions of the solubilized peptides were reacted overnight at 4 °C with MAb 7C2 or polyclonal serum before proceeding with the neutralization or plaque reduction assays as above. Blocking activity was defined as the complete reversal of neutralization or 50% plaque reduction activity.

**Polyclonal and monoclonal antibodies.** These two immunological probes have been characterized previously (Trudel et al., 1986). Briefly, virus-specific serum was produced in guinea-pigs by repeated intranasal infection (approx. 10° p.f.u.) to obtain specific antibodies to the virus. The polyclonal serum had a specific neutralizing titre of 1/128 and a 50% plaque-reducing activity of >1/17384. The MAb 7C2 had a neutralizing activity of 1/1024 and a plaque-reducing efficiency of >1/17384. Monoclonal antibody 11/C/12, specific for rubella virus, was used as control in the blotting experiments (Trudel et al., 1985).

**Peptide mapping.** The F1 fragment of the fusion protein was purified on 7-5% SDS–polyacrylamide gels and the appropriate band was cut out. For enzymic cleavage, 25 μg of the purified protein was digested with 10 μg of V8 staphylococcal protease, specific for the glutamyl residues (Drapeau et al., 1972; Austen & Smith, 1976). Digestion of the protein was carried out during electrophoresis in the stacking gel and resolved by continuing the electrophoresis on a linear 15 to 20 ~ SDS-polyacrylamide gel (Cleveland et al., 1977). For chemical digestion with N-chlorosuccinimide (NCS; Sigma), which cleaves at tryptophan residues (Lischwe & Ochs, 1982), 25 μg of protein was cleaved in the gel by incubation for 30 min with 15 μg-NCS in urea/H2O/CH3COOH (1 g/1 ml/1 ml). The gel slice was then washed with water and equilibrated in 10% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 3% (w/v) SDS and 625 mM-Tris–HCl, pH 6.8, before being loaded on the separating gel. For peptide visualization, the gels were silver-stained according to the method of Merril et al. (1980).

**Immunoblotting.** Cleaved peptides separated by SDS–PAGE were blotted (Western blot) onto nitrocellulose sheets (Bio-Rad) in transfer buffer, consisting of 20% (v/v) methanol added to the electrophoresis buffer of Towbin et al. (1979). Transfer was carried out at 50 V for 50 min using a Bio-Rad Trans-Blot cell. Synthetic peptides were dot-blotted onto nitrocellulose strips using the Bio-Dot apparatus (Bio-Rad). In both cases, non-specific adsorption sites on the blotted nitrocellulose strips were saturated by incubation for 30 min at 25 °C with 10 ml of blocking solution [1-5% (w/v) bovine serum albumin (BSA) in 50 mM-Tris–HCl, 150 mM-NaCl at pH 7.4 (TBS buffer)]. Strips were then washed in TBS buffer and incubated under mild agitation for 2 h at 25 °C, with 10 ml of guinea-pig serum specific for RS virus or with mouse ascitic fluid diluted 1/100 in TBS, containing 0-1% (v/v) Tween 20 non-ionic detergent (TBS-T buffer). The nitrocellulose strips were then washed three times in TBS-T buffer and further incubated for 2 h at 25 °C with 10 ml of a 1/2000 dilution of peroxidase-conjugated Protein A (Flow Laboratories). Specific immunoblotted bands were revealed with 0-05% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (Baker, N.J., U.S.A.) in TBS buffer with 0-1% (v/v) H2O2 (30%); the reaction was stopped by washing in distilled water when the desired intensity was reached.
Synthetic neutralization epitope of RSV

Localization of the neutralization epitope. Possible location of the epitope involved in neutralization was identified using the Kyte & Doolittle (1982) hydropathy profile of the linear amino acid sequence given for the A2 strain of RS virus (Elango et al., 1985) and the determination of β-turns by the application of the Chou & Fasman (1974) rules for the prediction of protein conformation, in conjunction with peptide cleavage data and preliminary amino acid composition of the immunologically active protein fragments.

Synthetic peptides. Peptides were synthesized on a p-alkoxybenzyl alcohol resin (IAF BioChem International Inc., Laval, Quebec, Canada) using N-9-fluorenylmethoxycarbonyl-amino acid derivatives. Each amino acid was coupled by the dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) method. Completeness of coupling was monitored with the ninhydrin test (Kaiser et al., 1970). The protecting groups used were tert-butyl (t-Bu) for Glu, Ser and Thr, tert-butyloxycarbonyl (t-Boc), tosyl and p-methoxybenzyl for Lys, Arg and Cys residues respectively. The N-9-fluorenylmethoxycarbonyl group was cleaved by 20% piperidine in N,N-dimethylformamide between couplings. Cleavage of the peptide from the resin and removal of the t-Bu and t-Boc protecting groups were done by treatment with 55% trifluoroacetic acid in methylene chloride in the presence of 5% anisole. The remaining protecting groups were cleaved by HF treatment for 60 min at 0 °C in the presence of 5% anisole and 5% ethanedithiol as scavengers. The composition of the synthetic peptides was confirmed by amino acid analysis and is given in Fig. 4: they span from Cys-216Asn to 232Glu (peptide 1), 221Cys to 222Glu (peptide 2) and Cys221·Ile to 236Glu (peptide 3).

RESULTS AND DISCUSSION

Peptide mapping of the F1 fragment of the fusion glycoprotein of RS virus with NCS and V8 staphylococcal protease yielded several immunoreactive fragments. Chemical cleavage with NCS yielded four fragments of 30K, 26K, 23K and 3.4K Mr, with the 23K fragment retaining the reactive neutralization epitope to MAb 7C2 (Fig. 1). Enzymic digestion with V8 protease yielded several fragments ranging from 21K to 7K, with an 11K fragment retaining the neutralization epitope. Smaller immunoreactive fragments of 10K, 8K and 7K were also observed, but their overall reactivity with MAb 7C2 was reduced (Fig. 2). As for the larger 21K to 14K fragments, they were incompletely digested peptides containing the 7C2 epitope.

A map of potential NCS and V8 cleavage sites of the F1 fragment of the fusion protein of RS virus was drawn based on the mRNA-deduced amino acid sequence of the A2 strain (Elango et al., 1985; Fig. 3). Two tryptophan residues are found on the protein at positions 314 and 341, permitting NCS to cleave the molecule completely into three fragments of 177, 27 and 233 amino acid residues having a calculated Mr of 23·2K, 3·7K and 30·5K respectively. This closely
resembled the pattern observed with NCS cleavage of the Long strain (30.5K, 26.4K, 23K and 3.4K), where the 26.4K fragment could be a partially cleaved fragment (23K + 3.4K). Immunoblotting revealed that only the 23K fragment retained the neutralizing epitope, suggesting that it was located within the region of residues 137Phe to 314Trp, in the first third of the F1 protein. One would also expect the 26.4K fragment to carry the neutralization epitope; perhaps the additional 3.4K fragment causes interference with the binding of the neutralizing MAb 7C2.

The proteolytic map of A2 F1 fusion protein with V8 protease also yielded data supporting our hypothesis (Fig. 3). Only two regions could possibly yield proteolytic fragments that could be large enough (11K and 7K) to agree with the peptide mapping data and to carry the neutralization epitope: these regions were located between residues 161Glu to 236Glu and 378Glu to 463Glu. The two terminal regions of the F1 protein, containing the hydrophobic domains, were excluded since their composition probably prevented them from being accessible at the surface of the protein. Since NCS mapping had already identified the first third of the molecule, we followed the hypothesis that the epitope was probably in the region of residues 161Glu to 263Glu, bearing in mind that there could be some variations in the Long strain sequence that did not occur in the A2 strain. However, since enzymic degradation was incomplete (Fig. 2, lanes 4 and 5) it was also possible that several combinations of adjacent fragments (Fig. 3) adding up to 7K or 11K may have contained the epitope. In themselves, although indicative, these results were insufficient to draw a conclusion on the location of the epitope.

Given the preceding results, we focused on the prediction of the possible location of antigenic determinants using hydropathicity profiles and \( \beta \)-turn positions (Kyte & Doolittle, 1982; Chou & Fasman, 1974). It was noted that region 161Glu to 236Glu contained five predicted regions of \( \beta \)-turns beginning at positions 173Thr, 181Leu, 189Thr, 211Ser and 227Asn and that residues 215Ser to 226Lys plotted out as a strong hydrophilic region (Fig. 4).

Since the peptide mapping data and the hydropathicity profiles indicated the same regions, we next prepared a series of three synthetic peptides whose sequences were chosen within the 212Cys to 236Glu region. Peptide 1 (Cys-216Asn to 232Glu) gave a strong positive dot-blot signal (Fig. 5) with anti-RS virus guinea-pig serum (0.15 \( \mu \)g) and neutralizing MAb 7C2 (1.25 \( \mu \)g). Guinea-pig pre-immunization serum or MAb 11/C/12 were negative in the assay, so was a
Control peptide derived from human blood factor VIII blotted with a mixture (1 : 1, v/v) of MAb 7C2 and anti-RSV virus guinea-pig serum. These results showed the main neutralization epitope to be located between residues 215Ser and 232Glu, supporting our peptide cleavage data indicating that the epitope was in the first third region of the F1 protein. Peptide 2 (212Cys to 222Glu) and peptide 3 (Cys·221Ile to 236Glu) also confirmed this finding. Dot-blots of the three peptides with 7C2 (Fig. 6) showed a positive reaction at 0·31 µg of peptide 1, 10 µg of peptide 2 and 1·25 µg of peptide 3. These results suggested that the epitope was located mainly between residues Cys·221Ile to 236Glu (peptide 3). The lower level of reactivity shown with peptide 2 may have indicated that this peptide only carried a small portion of the epitope. The peptide derived from human blood factor VIII was negative in these assays.
Fig. 4. (a) Hydropathicity profile of the RS virus F1 subunit of the A2 strain (Elango et al., 1985) calculated according to Kyte & Doolittle (1982). (b) Detailed sequence of a presumed antigenic site identified by the expanded region (210Gln to 236Glu), giving possible β-turn locations, the sequences and relative positions of the three synthetic peptides that were prepared.

Fig. 5. Dot-blot of synthetic peptide 216Asn to 232Glu with pre-immunization (lane 2) and convalescent (lane 1) guinea-pig serum, RS virus-neutralizing MAb 7C2 (lane 3). Controls included rubella virus-neutralizing MAb 11/C/12 (lane 4) and a synthetic peptide derived from human blood factor VIII blotted with a mixture of MAb 7C2 and RS virus-positive polyclonal sera (lane 5).

The peptides were next assayed by neutralization and plaque-reduction assays. Peptides 1 and 3 (Cys·216Asn to 232Glu and Cys·221Ile to 236Glu), used at between 0.25 and 5 µg, were efficient in blocking neutralization by polyclonal serum (guinea-pig anti-RS virus: neutralizing titre 1/128) and MAb 7C2 (neutralizing titre 1/1024). Peptide 2 (212Cys to 222Glu) was not able to block neutralization and neither was the control peptide derived from blood factor VIII. Peptides 1 and 3 were also efficient in blocking the 50% plaque reduction assay. The plot of peptide concentration against blocking of neutralization (plaque reduction assay with polyclonal serum) showed that only peptides 1 and 3 were efficient, with similar slopes (Fig. 7).

From the preceding results, it could not be concluded that peptides 1 and 3, containing only one neutralizing epitope, adsorbed all the neutralizing activity of the polyclonal serum. The results were obtained at the 1/128 dilution of serum, which represented the last dilution at which
complete neutralization was observed. Several distinct neutralization epitopes on the fusion protein have been reported (Samson et al., 1986; Walsh et al., 1986; Trudel et al., 1987). Walsh et al. (1986), using 13 MAbs, have identified three neutralizing sites on the fusion protein. We have confirmed this observation and have added a fourth neutralization site (Trudel et al., 1987). However, only one of these MAbs (MAb 7C2) completely neutralized the virus at a high dilution (1/1024), indicating that the corresponding epitope was a major neutralizing epitope. In the 50% plaque reduction assay, MAb 7C2 was efficient at a dilution equal to or superior to 1/17384. Several MAbs against the three other neutralizing epitopes were negative in neutralization and showed only 50% plaque reduction activity in the range of 1/32 to 1/2048, which was at least 10-fold inferior to the other neutralization epitopes on F1 (Trudel et al., 1987). Furthermore, MAbs to the three neutralizing epitopes reported by Walsh et al. (1986) were only tested at 1/100 dilution in a 50% plaque reduction assay, not permitting the differentiation of major and minor neutralization epitopes; however, we suspect that MAb L4, which had neutralizing and fusion-inhibiting activities and blotted with the F1 fragment, just like our MAb 7C2, might react with the major epitope. These results suggested that although there are several neutralizing epitopes on the fusion protein of RS virus, the one which we had previously identified contributes much more than the others, qualifying it as a major neutralizing epitope. It is thus possible that a peptide containing this epitope could reverse the neutralizing capacity of a polyclonal serum as well as MAb 7C2, since for the most part, the neutralizing antibodies of polyclonal serum were directed to this epitope.

These results support our hypothesis that a very significant portion of one of the neutralization epitopes found on human, bovine and caprine strains of RS virus is found on the dodecapeptide -Ile-Glu-Phe-Gln-Gln-Lys-Asn-Asn-Arg-Leu-Leu-Glu-. It also demonstrates that this epitope can be efficiently mimicked by a short linear sequence, and opens a promising avenue for the simple design of a synthetic vaccine to control RS virus infection.

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